CHARACTERIZATION OF AMYLOSE LIPID COMPLEXES AND THEIR EFFECT ON THE CORN DRY GRIND PROCESS

BY

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THESIS

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ABSTRACT

The dry grind process is the most commonly used method for production of corn ethanol. Ethanol from corn is the biggest contributor to bioethanol produced in the US. In this study, effects of different parameters such as liquefaction temperature, solids content and particle size on formation of amylose lipid complexes (AML) were observed. This helps determine better methods of application in the dry grind industry by providing reasons for decreasing available starch.

In the conventional dry grind process, starch in corn is liquefied to dextrins at high temperature, followed by simultaneous saccharification and fermentation (SSF), where dextrins are converted to monomers and are fermented simultaneously to ethanol by yeast. Prior to liquefaction, there is 73% starch available for enzymatic hydrolysis in ground corn. However, after liquefaction the available starch content decreases to 61%. This study aimed to identify decrease in available starch as being due to formation of AML.

The ethanol concentration from utilizing granular starch hydrolyzing enzymes (GSHE), which is carried out at a constant temperature without a liquefaction step, is comparative to a conventional dry grind process. However, distillers' dried grains with solubles (DDGS) obtained from the GSHE process contained 24% residual starch compared to a much lower 10% from the conventional process. This indicated that in addition to the loss of glucose to other streams during fermentation there was available starch lost during the liquefaction step. Addition of lipids to starchy food alters the physical and chemical composition due to formation of AML at high temperatures above 80ºC. These complexes decrease the water solubility and susceptibility of the starches to α-amylase digestion.

AML content was found to decrease from 3.5 to 1.0% in post liquefaction solids (liquefact) as corn grind size was increased 0.5 to 2.5 mm. Across all solids content tested (25, 32 and 34%), the mean AML content was 0.61% lower when liquefaction temperature was increased to 105ºC, relative to the 85ºC liquefaction temperature. At 85ºC, liquefact from all three α-amylases, had similar AML content. However, when liquefaction temperature was increased to 105ºC, liquefact from enzyme AA2 had the lowest AML formation compared to other amylases.

A differential scanning calorimetric (DSC) study was carried out to identify the formation of AMLs during liquefaction. The phase transitions that starch undergoes, including starch gelatinization and crystallization of AMLs, were identified in thermograms obtained from the DSC study. A starch standard containing soluble starch and linoleic acid, was made to undergo liquefaction. There was a 5% decrease in available starch content after liquefaction. In a control in which linoleic acid was taken with the same solid content and kept in an incubator to replicate a GSHE process without high temperature resulted in no change in available starch content. This confirmed AML formation during liquefaction in the dry grind process.

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Chapter 1

INTRODUCTION

In the US, corn ethanol is the most commonly used form of bioethanol (EIA, 2017). About 143 million metric ton of corn produced in the US was estimated to be utilized for dry grind ethanol processing in 2019 (USDA, 2019). The dry grind process utilizes starch from whole ground corn to produce ethanol. Whole corn is ground in a hammer mill and passed through a sieve to achieve desired grind size of 0.5 to 2.5 mm (Naidu et al., 2007; Rausch et al., 2005). The desired solids content is achieved by adding water to form a slurry (Humbird et al., 2011; Kadhum et al., 2017; Mussatto et al., 2010). The slurry is treated at high temperatures (≥ 85ºC) for 1 to 2 h to gelatinize starch, also referred to as cooking (Bothast and Schlicher, 2005; Lee and Kim, 1990). Thermostable α-amylases are used to hydrolyze starch. The thermostable α-amylases have an optimal usage temperature of 80 to 110ºC. Postliquefaction solids (liquefact) are cooled followed by addition of glucoamylase to break down the long chain dextrins into smaller fermentable sugars. Fermentation of sugars is carried out simultaneously with saccharification by adding saccharification enzyme in a process called simultaneous saccharification and fermentation (SSF). SSF is carried out at 32ºC for 48 to 72 h for complete conversion of sugars to ethanol (Figure 1.1). Cooking and liquefaction of corn is the most important part of industrial dry grind operation as it determines the sugar available for fermentation by yeast and hence, influences fermentation yield.

High temperature cooking allows for starch gelatinization; which destroys starch granular structure and changes its properties, such as granular swelling, native crystallite melting and starch solubilization (Ellis et al. 1998; Morrison 1995). Gelatinization of corn starch occurs over a range of temperature because energy required for molecular disruption of starch varies

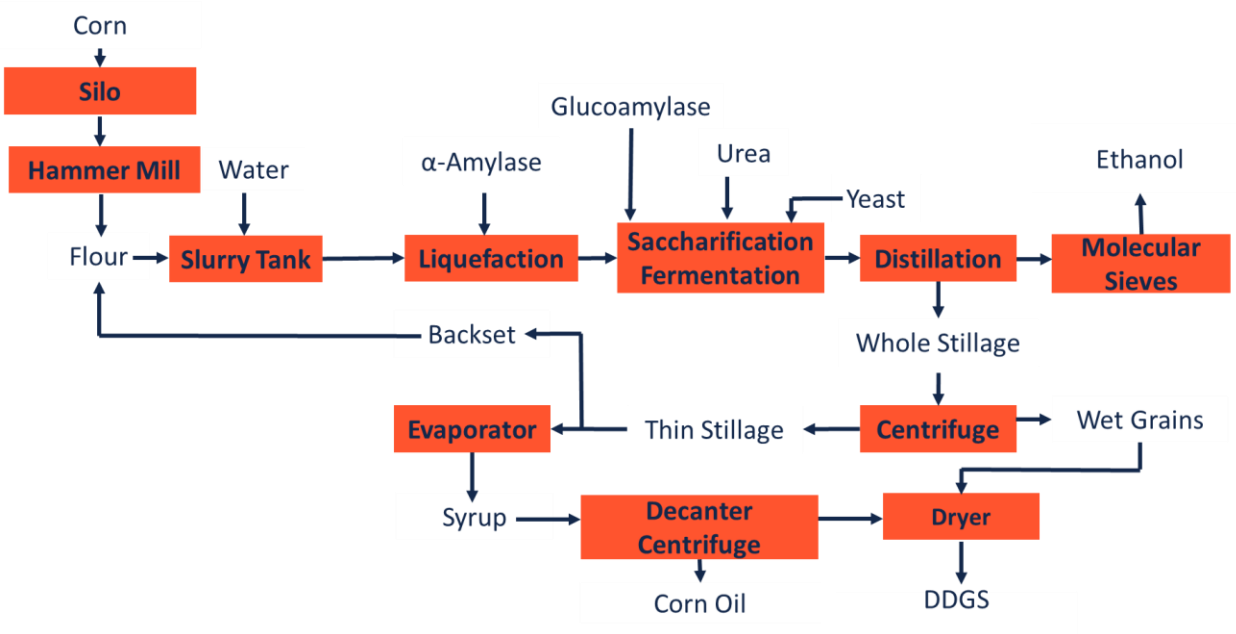


Figure 1.1. A schematic of the dry grind process showing grinding, slurrying and liquefaction. As well as, SSF, ethanol purification and coproduct extraction processes.

depending on individual granules in cereal. Grinding of corn is carried out prior to gelatinization, as mechanically broken starch granules allow release of long chain dextrins at a lower temperature compared to intact granules (Lynn and Stark, 1995). Accessibility of starch to enzymes (α-amylase) for starch breakdown depends on the particle size of corn used for liquefaction. Complete gelatinization occurs only in the presence of excess water (Atwell, 1988) and a high solids content would prove detrimental to the liquefaction process. Thus, major factors affecting liquefaction are temperature, solids content and particle size (Kwiatkowski et al., 2006; Lynn and Stark, 1995; Morrison, 1995).

Starch granules are made up of amylose and amylopectin as constituent alpha glucans. Structural complexity is affected by variations in moisture, lipid and protein content. Amylose (MW 10^5 to 10^6) is a long chain, linear alpha glucan containing α (1-4) linkages. Amylopectin (MW 10^7 to 10^9) is larger and has higher molecular weight relative to amylose and has a branched structure

made up of mostly α (1-4) linkages and α (1-6) linkages (Atwell, 1988; Ellis et al., 1998; Tester and Morrison, 1990). The amylose to amylopectin ratio in cereal grains affects gelatinization temperature and hence the liquefaction process. Corn starch used for bioethanol contains a higher ratio of amylopectin to amylose and a lower content of lipids.

Due to structural characteristics of corn starch (linear chain with α (1-4) bonds), and noncrystalline nature, amylose can interact with endogenous lipid molecules forming amylose lipid complexes (AMLs) (Ellis et al., 1998; Cervantes-Ramírez et al., 2020). The length of fatty acid chain restricts interactions between amylose and lipids but during gelatinization; double helix of amylose opens, allowing fatty acids to interact and form stable hydrogen bonds. The capacity to form hydrogen bonds, as well as hydrophobicity of fatty acid ligands are factors that affect formation of these complexes. AMLs are an inclusion complex that generate a left handed structure that deforms ligand structure leading to low reactivity, which in turn affects the starch behavior, changing its pasting properties, water absorption capacity, solubility, swelling capacity and viscosity (Becker et al., 2001). AMLs are characterized as resistant starch that cause functional modification in starch behavior (Cervantes-Ramírez et al., 2020). Kaur et al. (2000) and Cervantes-Ramírez et al. (2020) have reported changes in the physical and chemical composition of starch during high temperature extrusion of cereals and grains in the presence of lipids (Cervantes-Ramírez et al., 2020; Kaur and Singh, 2000). Formation of resistant starch and subsequent changes in starch properties, is attributed largely to formation of AMLs at a high temperature in the presence of excess water. AMLs decrease water solubility of starch and susceptibility of starches to α-amylase enzymes (Nebesny et al., 2005; Seneviratne and Biliaderis, 1991). AMLs are thermally unstable and dissociate at temperatures from 100 to 135ºC; however, they reform easily on cooling (Kugimiya et al., 1980; Lee et al., 2020).

AMLs are likely to form during liquefaction, under high temperature and excess water, due to interaction between gelatinized starch and lipids. Since starch bound to fatty acids in AMLs is inaccessible to α-amylases, it would affect total sugar available for fermentation. This unavailable starch has implications on the corn ethanol industry where every percentage increase in ethanol output affects the economic return. Starch not utilized during fermentation will end up in low value distillers' dried grains with solubles (DDGS) which constitute a major coproduct of corn ethanol production.

Analysis of these DDGS to estimate residual starch does not account for lipid bound, complexed starch molecules (AML), as starch estimation is based on enzyme hydrolysis to which AMLs are resistant (Figure 1.2). An observable change in the starch available in preliquefaction corn slurry compared to postliquefaction solids (liquefact), is indicative of AML formation. Analysis of liquefact for starch available for enzymatic hydrolysis does not account for these starch molecules bound in AMLs as well. AMLs in liquefact are not measured because the enzymatic assay used for starch estimation is not able to hydrolyze AMLs (Becker et al., 2001; Nebesny et al., 2004). Knowing that AMLs are thermally unstable (Kugimiya et al., 1980; Lee et al., 2020), total starch estimation for liquefact and DDGS was carried out by dispersing samples in dimethyl sulfoxide (DMSO) at high temperatures (> 100ºC). AMLs break at high temperature, which frees the lipids. The released lipids are dissolved in DMSO. Starch molecules which were bound in AMLs are now available for enzymatic reactions. Breaking down AMLs and releasing the starch allows accurate determination of starch bound by AMLs (Srichuwong and Jane, 2011). Difference between total starch measured from enzymatic estimation after sample was dissolved in DMSO; and available starch estimated from standard enzymatic assay, was determined and was indicative of starch bound to AML.

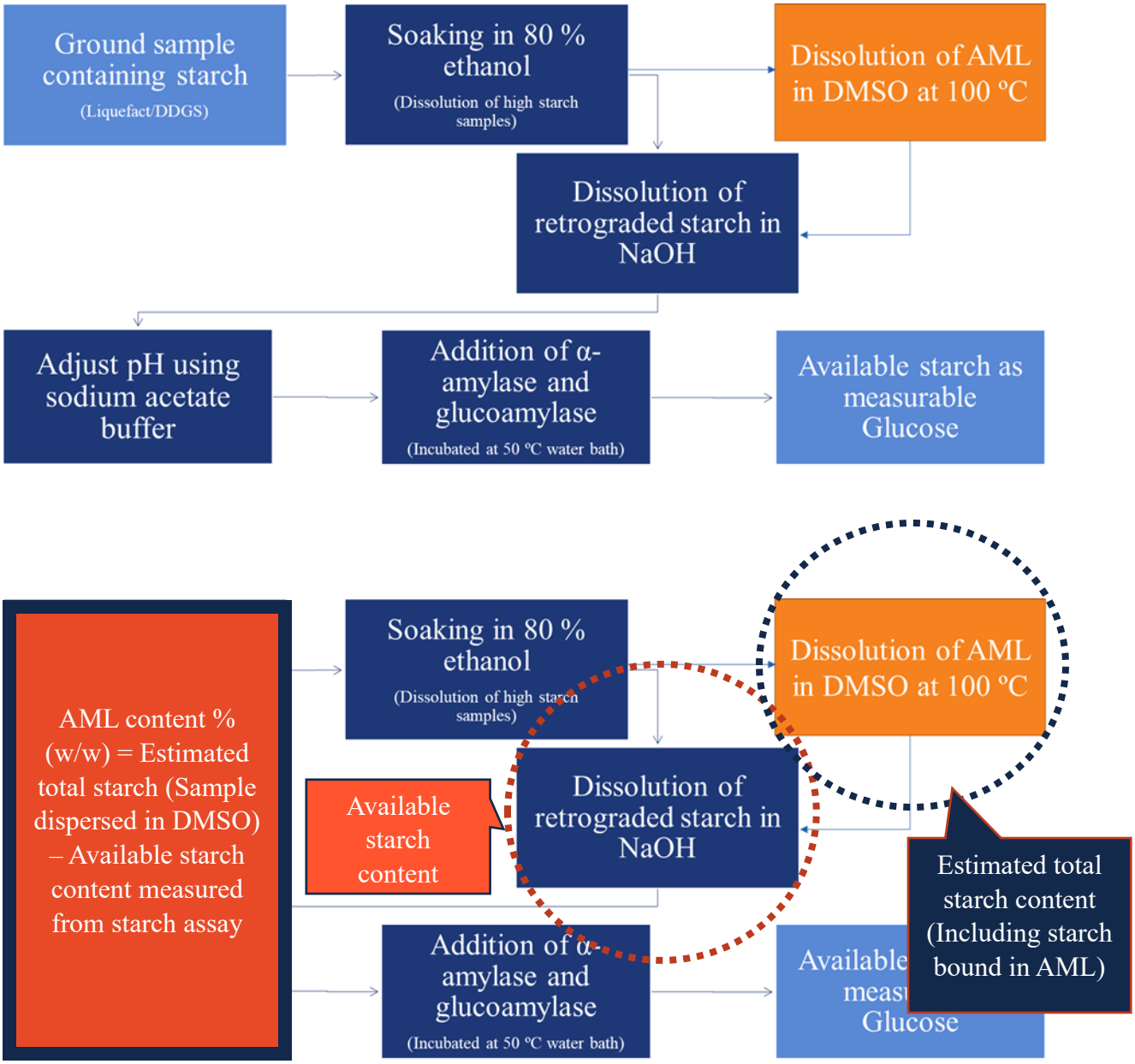


Figure 1.2. Schematic illustration of enzymatic starch estimation method. Dissolution of sample in DMSO allows estimation of resistant starch by the standard enzymatic method.

Another way to characterize AMLs is by using differential scanning calorimetry (DSC). Studies have been conducted to observe phase transitions that starch undergoes on heating in the presence of water and lipid. Thermograms showed gelatinization endotherms, followed by exothermic transitions at temperatures higher than gelatinization temperature (Kugimiya et al.,

1980; Nebesny et al., 2002). This exothermic enthalpic change of bond formation is an indication of AML formation.

AML formation during liquefaction in the corn dry grind process, and the effect of varying process parameters on AML content in liquefact, has not been studied systematically. Hence, the objectives of this study were to:

1. Determine effects of liquefaction parameters such as temperature, solids content percentage (w/w), corn particle size and different commercial α-amylases on AML formation in the dry grind ethanol process.
2. Observe and characterize change in starch content as being due to AML formation.
3. Ascertain combined effects of liquefaction parameters on lowering AML formation in the dry grind process.

Chapter 2

REVIEW OF LITERATURE

Cereals are major commercial sources of starch and corn is the most widely available cereal in the US. Corn starch that is broken down by enzymatic reaction and fermented by yeast yields bioethanol.

2.1 Corn starch and lipid

Corn starch is stored in starch granules inside the corn endosperm enmeshed in a protein matrix (Rooney and Pflugfelder, 1986). The starch polysaccharide is composed of glucose units. Starch is a glucan made up of two major types of molecules, amylose (linear chain) and amylopectin (branched chain). Amylose and amylopectin have differing arrangements of glucose molecules. Corn starch has 75% amylopectin content (Rooney and Pflugfelder, 1986).

2.1.1 Amylose and amylopectin

Amylose and amylopectin molecules are 98 to 99% of starch dry weight (Tester et al., 2004). The amylose content in normal cereal starches is 20 to 30%, but waxy starches contain little or no amylose (Rooney and Pflugfelder, 1986). Structures of amylose and amylopectin have been discussed by many authors (Atwell, 1988; Ellis et al., 1998; Rooney and Pflugfelder, 1986; Tester et al., 2004). Amylose is a linear polymer with long chains of up to 6000 glucose units connected with α (1-4) linkages and a molecular weight of up to 2×10^6 (Rooney and Pflugfelder, 1986). Amylopectin is a branched polymer made of short segments of 10 to 60 glucose molecules with α (1-4) linkages and side chains of 15 to 45 glucose units with α (1-6) linkages. Molecular weight of amylopectin ranges from 10 to 500×10^6 (Herrero-Martínez et al., 2004) and may contain up to 2 million glucose units arranged in branched clusters.

Due to differing physical structure and chemical bonding, amylose and amylopectin exhibit different functional properties especially when suspended in water (Klucinec and Thompson, 2002; Tester and Morrison, 1990). Linear amylose rapidly aligns with other linear chains when in solution which results in extensive hydrogen bonding and higher bond strength. Thus, these complexes have high dissociation energy and are difficult to break (Ellis et al., 1998). These complexes also increase the energy requirement for starch gelatinization and are resistant to external chemical action. Amylopectin molecules are branched and cannot align as easily as amylose; therefore, have weaker hydrogen bonding. The higher molecular weight of amylopectin due to its highly branched structure is responsible for higher viscosity (Tester et al., 2004).

Hence, high amylose starches will lead to formation of resistant starches and high amylopectin starches will give high viscosity (Rooney and Pflugfelder, 1986). The amylose to amylopectin ratio in cereal affects the gelatinization temperature, hence the liquefaction process.

2.1.2 Corn lipid

Corn lipids are stored in the germ and between the starch matrix (Hahn and Hood, 1987). Corn oil in the germ varies in unsaturation. Oil contents and fatty acid profiles of corn lipids vary based on geography of origin, hybrid and method of detection used (Beadle et al., 1965; Lofland et al., 1954). Corn contains 42 to 56% linoleic acid, as determined by iodine value (Beadle et al., 1965). Other commonly present fatty acids are palmitic acid, stearic acid, oleic acid and linolenic acid (Moreau et al., 2011).

2.2 Corn starch liquefaction

Starch acts as a source of fermentable sugars. In native starch, glucose is nested in a complex crystalline structure which is insoluble in water. Liquefaction of starch breaks down the

crystalline complex and makes starch more water soluble. Enzymatic liquefaction of corn starch is the most commonly used method of starch liquefaction for industrial products. In the conventional dry grind process, starch is cooked to gelatinize at high temperatures and hydrolyzed by using amylase enzymes (Jacques et al., 2003) in the liquefaction step.

2.2.1 Corn starch gelatinization

Starch granules have crystalline and amorphous polymeric regions (Abd Karim et al., 2000). Crystallinity is imparted by organization of amylopectin and the amorphous structure is attributed to amylose, which is distributed randomly among amylopectin arrangements (Ellis et al., 1998; Rooney and Pflugfelder, 1986; Tester et al., 2004). Starch is insoluble in cold water but in warm water, starch gelatinizes and loses its crystalline structure. During gelatinization, warm water is absorbed into the starch molecule which lowers hydrogen bonding. High temperature and excess water cause amylose to diffuse into the solution, increasing viscosity and further loosening hydrogen bonds.

The purpose of cooking the corn slurry is for solubilization of sugars, to release the sugars and dextrins bound in protein and fiber and to reduce the slurry viscosity so that it can be pumped for further processing. Starch gelatinization temperature depends on amylose content and requires slurrying with water (Atwell, 1988; Ellis et al., 1998; Jacques et al., 2003).

2.2.2 Enzymatic breakdown of starch

Industrial starch based products are manufactured most widely by enzymatic hydrolysis (Ellis et al., 1998). Depending on the end product required, different enzymes can be used for starch breakdown. Alpha-amylase is an endo acting enzyme that hydrolyzes starch linkages to form linear and branched oligosaccharides. It is used in tandem with exo acting enzymes such as

glucoamylase to hydrolyze starch completely to monosaccharides and oligosaccharides which can be metabolized by yeast during fermentation.

2.3 Factors affecting starch liquefaction

Liquefaction is governed by starch granule size, shape and amylose content (Tester et al., 2006). Increased surface area to volume ratio of starch allows faster enzyme hydrolysis due to larger surface area for enzymes to attach. As size and shape of starch granule varies based on source, α-amylase activity will vary based on starch source as well.

Amylose content is correlated negatively to ease of liquefaction (Klucinec and Thompson, 2002; Oates, 1997). Amylases are absorbed more readily onto corn starch granules with high amylopectin content compared to normal corn starch but rate of solubilization is similar (Oates, 1997). Presence of resistant starch formed due to amylose complexes also resist starch digestion (Haralampu, 2000).

2.3.1 Excess water

The presence of excess water is important in starch gelatinization as it plasticizes amorphous regions and creates a mobile phase allowing the crystalline regions to melt (Rooney and Pflugfelder, 1986). If water is limited, greater heat or mechanical energy is required to promote loss of organization of crystalline regions. High starch concentration, due to high slurry solids content, impedes starch gelatinization. Granular and crystalline structure is not destroyed above 45% solids content (Atwell, 1988; Jørgensen et al., 2007; Kadhum et al., 2017; Li et al., 2015).

2.3.2 High temperature

Heating starch in excess water causes the rapid swelling of starch granules. As swelling progresses and hydrogen bonds are broken, amylose leaches from the granules. The characteristic gelatinization temperature for starch is at the point there is 50% loss of birefringence (Rooney and Pflugfelder, 1986). As temperature is increased to reach 100ºC, the granules expand and lose their integrity. A layer of gelatinized starch might form an impervious layer preventing action of amylases. Therefore, corn slurry is heated to temperatures of 90ºC to gelatinize all the starch. Slurry is agitated to ensure that there is no increase in viscosity and to reduce loss of starch (Jacques et al., 2003).

2.3.3 Mechanical grinding

Starch granules may have a reduced gelatinization temperature if there is prior loss of crystallinity (starch granule damage) due to mechanical grinding. The energy requirement may be increased further if starch granule crystallites are allowed to anneal before gelatinization (Hoover and Vasanthan, 1994). Undamaged starch has low susceptibility to enzymatic attack. Damage to starch due to mechanical grinding of grain, decreases with an increase in corn moisture content and increases with increased milling speed.

2.4 Resistant starch

Not all starch is converted to simple sugars during enzymatic starch hydrolysis. The starch fraction that remains unreacted is called residual starch. The amount of residual starch left after hydrolysis depends on process parameters such as temperature, time, solids content and enzyme action. A portion of this residual starch is enzyme resistant and acts like crude fiber and is called resistant starch. This term was defined as starch fraction which is not hydrolyzed by the small

intestine and escapes to the large intestine for digestion (Englyst and Hudson, 1987; Englyst et al., 1982; Englyst and Cummings, 1985).

Susceptibility of starch to enzymatic digestion is affected by varying factors based on which they are classified into four classes; RS1: physically inaccessible to digestion due to entrapment in a nondigestible matrix; RS2: ungelatinized starch; RS3: retrograded starch; RS4: chemically modified starch (Haralampu, 2000).

Amylose content is correlated positively to residual starch due its tendency of forming thermally stable, enzyme resistant compounds, owing to its linear structure (Haralampu, 2000). Waxy starch forms less resistant starch compared to high amylose starch, but it is not possible to predict enzyme susceptibility of corn by amylose content alone (Klucinec and Thompson, 2002).

Processing high amylose starch with free fatty acids forms novel resistant starch classified as RS5: amylose lipid complexes (Hasjim et al., 2013). It is classified as a separate class as it has a high content of slowly digesting starches compared to resistant starches. These RS5 are known to breakdown over long period of time (72 h) in vivo during enzymatic hydrolysis (Nebesny et al., 2002; Hasjim et al., 2013; Lee et al., 2020).

2.5 Amylose lipid complexes

Amylose chains, as well as exterior chains of amylopectin, can form double helices which may associate to form crystalline domains with endogenous lipids present in cereal. This leads to formation of amylose lipid complexes (AML), a kind of resistant starch (Amoako and Awika, 2019; Hahn and Hood, 1987). Lipid fractions within starch granules are insufficient to saturate the amorphous amylose fraction and form fully saturated amylose lipid complexes (Ellis et al., 1998; Gelders et al., 2005).

Interaction of noncrystalline amylose molecules with lipids to form inclusion complex is restricted only by length of amylose chain. The capacity to form hydrogen bonds and hydrophobicity of the ligand are other factors affecting AML formation. Amylose lipid complexes are formed due to interaction of fatty acids with amylose double helices. These complexed amyloses generate a left handed structure leading to low digestibility and resistant nature of these complexes (Cervantes-Ramírez et al., 2020; Hasjim et al., 2013).

2.5.1 Relevance

In the food processing industry, steam jet cooking of high amylose starch and fatty acid mixtures is known to lead to formation of AML (Dintzis and Fanta, 1996; Fanta et al., 1999; Kaur and Singh, 2000). These complexes also are known to be formed during extrusion cooking of starch lipid mixtures (Bhatnagar and Hanna, 1994; De Pilli et al., 2008).

Lipids form inclusion compounds with amylose leading to reduced water solubility and susceptibility of these starches to enzymatic digestion. Moreover, this functional modification of starch, changing its pasting properties, retrogradation, change in solubility and swelling capacity are induced by amylose lipid complexes (Bhatnagar and Hanna, 1994; De Pilli et al., 2008; Kaur and Singh, 2000). The formation of resistant starch (Hasjim et al., 2013) which is impervious to enzymatic breakdown can change the viscosity of cereal starch slurry.

Resistant starch from AML has novel applications in the food industry (Lee et al., 2020), as a textural component in food and as a healthier substitute for shortening and oil. However, formation of these complexes in high temperature liquefaction during conventional dry grind ethanol process might impede efficient hydrolysis and fermentation. Formation of AML during starch hydrolysis and the effect that enzymatic hydrolysis has on AML has been studied for

enzymatic hydrolysis of wheat starch to glucose (Nebesny et al., 2002), including the effects of changes in hydrolysis conditions on AML formation (Nebesny et al., 2004; 2005).

2.5.2 Characterization of amylose lipid complexes

Different fatty acids, cooking temperature and their effects on extent of complex formation and the corresponding effect on properties like starch morphology, viscosity, structure and thermal properties have been studied (Bhatnagar and Hanna, 1994; Cervantes-Ramírez et al., 2020; De Pilli et al., 2008; Kaur and Singh, 2000; Obiro et al., 2012).

Amylose lipid complexes are formed by heating starches with lipid and water. The complexes are formed in an exothermic reaction that occurs as soon as starch gelatinizes. It is difficult to determine whether these complexes preexist in native starches or are formed upon heating above gelatinization temperature. The extent of complex formation depends on amylose content and type of lipid and has been studied via differential scanning calorimetry (DSC), iodine complexing index and X-ray diffraction (Bhatnagar and Hanna, 1994; Kugimiya et al., 1980; Lee et al., 2020; Seneviratne and Biliaderis, 1991).

Quantitative measurement of crystalline order using DSC, measures gelatinization enthalpy (ΔH). Calorimetric studies of starch with added lipid and water show AML formation upon gelatinization as there is an exotherm (indicating evolution of heat due to crystalline formation) simultaneous with, and immediately following, the starch gelatinization endotherm. This exotherm is prominent when thermograms of pure starch gelatinization are compared to it, as a major portion of the exotherm for formation of amylose lipid complex is superimposed on the high temperature arm of the gelatinization endotherm (Kugimiya et al., 1980).

Another thermal transition is seen around 100ºC when heated with large amounts of lipid in large quantities of water. This endotherm is indicative of dissociation of complex. However, this transition is heat reversible and the complex can form again on cooling (Eliasson and Ljunger, 1988; Kugimiya et al., 1980). AML formation is seen only for water soluble fatty acids. Lipid content in pure starch is 1% or less; therefore, this thermal transition is not observed in the thermogram of heating pure starch. The thermal transition characteristic to the AML confirms that it is formed immediately on heating after gelatinization (Kugimiya et al., 1980).

2.5.3 Measurement of amylose lipid complex content

Starch determination is carried out by using the gelatinization of starch at elevated temperature in the presence of thermo stable α-amylase to release linear and branched dextrin components. These are quantitatively hydrolyzed to glucose with amyloglucosidase (Baur and Alexander, 1979; McCleary et al., 1994a; McCleary et al., 1994b). The released glucose is measured using high performance liquid chromatography (HPLC) or other glucose determination methods.

AMLs are enzyme resistant and not detected by standard enzymatic starch determination methods. AMLs have high dissociation temperatures between 95 to 135ºC (Kugimiya et al., 1980); therefore, heating in boiling water cannot disperse these crystalline resistant starches. Dimethyl sulfoxide (DMSO) is a hydrogen bond acceptor and a powerful solvent that can dissociate starch single and double helices. DMSO is used to dissolve AML crystals to free starch molecules that are susceptible to enzymatic hydrolysis (Srichuwong and Jane, 2011). This method was developed to determine AML bound resistant starch in DDGS.

Chapter 3

CHARACTERIZATION OF AMYLOSE LIPID COMPLEXES AND THEIR EFFECTS ON THE DRY GRIND PROCESS

3.1 Introduction

Liquefaction (cooking and hydrolysis) of corn is one of the most important parts of industrial dry grind operation as it affects total sugar available for fermentation by yeast and influences fermentation output (Ellis et al., 1998; Morrison, 1995). Major factors affecting liquefaction are temperature, percentage (w/w) solids content and corn particle size (Bothast and Schlicher, 2005; Jacques et al., 2003; Kadhum et al., 2017; Kwiatkowski et al., 2006; Lynn and Stark, 1995; Morrison, 1995).

Changes in physical and chemical behavior of starch happen due to the formation of amylose lipid complexes (AMLs) at high temperatures, in the presence of excess water. Lipids present in ground cereal grains, lead to the formation of AMLs (Nebesny et al., 2002). AMLs are resistant starch that cause functional modification in the physical and chemical behavior of starch (Cervantes-Ramírez et al., 2020; Haralampu, 2000). Starch bound lipid complexes are unstable thermally and break at temperatures between 100 and 135ºC; however, they reform readily upon cooling (Kugimiya et al., 1980; Lee et al., 2020). AMLs also affect gelatinization and swelling of starch (Cervantes-Ramírez et al., 2020; Hasjim et al., 2013; Seneviratne and Biliaderis, 1991; Tester and Morrison, 1990). It is likely that AMLs also are formed under high temperature and excess water conditions during liquefaction of ground corn in the dry grind process (Dintzis and Fanta, 1996; Haralampu, 2000; Sharma et al., 2010). These complexes decrease water solubility and susceptibility of starches to α-amylase enzymes (Becker et al., 2001; Nebesny et al., 2002;

2005; Seneviratne and Biliaderis, 1991). Formation of AMLs would affect total sugar available to yeast for fermentation. AMLs are not broken down into fermentable sugars and would end up in DDGS, reducing ethanol yield (Jacques et al., 2003) and impacting profitability of the dry grind process.

Observable change in available starch in preliquefaction corn slurry compared to postliquefaction solids (liquefact) is indicative of AML formation. Analysis of liquefact for available starch for fermentation does not account for these complexed starch molecules because the enzymatic assay used for starch estimation is not able to hydrolyze these AMLs (Becker et al., 2001; Nebesny et al., 2004). Liquefact sample was dispersed in dimethyl sulfoxide (DMSO), a lipid solvent, and temperature was increased to > 100ºC. Thermally unstable AMLs break down (Kugimiya et al., 1980; Lee et al., 2020), and the DMSO solubilizes lipids. Thus, making free starch molecules available for enzymatic reaction. Breaking down AML and releasing the starch allowed us to determine accurately starch complexed in AMLs (Srichuwong and Jane, 2011). The difference between estimated total starch, measured after DMSO treatment of sample and available starch measured from standard enzymatic assay after liquefaction were determined and were indicative of starch complexed in AML. Specific objectives were to determine impact of liquefaction parameters such as temperature, solids content percentage (w/w), corn particle size and different commercial α-amylases on AML formation in the dry grind ethanol process.

3.2 Materials and methods

3.2.1 Materials

Ground corn was obtained from a commercial dry grind plant and stored at 4ºC in a plastic bucket. Corn from dry grind facility is commodity corn which is a mixture of different varieties.

This allows estimation of effect of AML formation on the dry grind process, without being biased by AML formation for an isogenic variety. The corn was re-ground at 500 rpm in a laboratory hammer mill (Retsch SK100, Glenn Mills, Clifton, NJ) and screened through a sieve attached to the mill. Samples were stored in Ziploc bags at 4ºC until analysis. Moisture contents of ground corn samples and DDGS were measured by drying weighed samples in a convection oven at 105ºC overnight (AACCI Approved Method 44-15.02, AACCI, 2020).

The α-amylases and glucoamylases used were commercial enzymes used in dry grind ethanol processing. The study was carried out using three thermostable α-amylases (recommended range 82 to 86ºC), AA1, AA2 and AA3 and one low temperature α-amylase, AA4, having activities of 11648, 13501, 11321 and 15217 µmol maltose/ml enzyme, respectively. The two amyloglucosidases, GA1 and GA2, with 18197 and 24194 µmol glucose/ml enzyme activities, respectively, were used for saccharification. A granular starch hydrolyzing enzyme (GSHE) having combined alpha and glucoamylase activities of 46653 µmol maltose/ml and 30347 µmol glucose/ml, respectively, also were used in this study. All α-amylase and glucoamylase enzyme activities were defined as reducing sugars produced per milliliter of enzyme solution used. The assay (µmol sugar/ml enzyme) used 3,5 - dinitro salicylic assay with pregelatinized potato starch and maltodextrins as substrate, respectively, incubated for five min (Ramchandran et al., 2016; Ramchandran et al., 2015).

Liquefaction experiments were carried out at optimal pH and temperature conditions provided by enzyme manufacturers. Ethanol Red conventional dry yeast was used for fermentation (Fermentis-Lessaffre Yeast Corporation, Milwaukee, WI).

3.2.2 Liquefaction

To determine the role of liquefaction parameters such as temperature, slurry solids content (w/w) and action of α-amylase, several liquefaction experiments were carried out as shown in Table 3.1. Throughout this text, % is defined as % (w/w) dry basis unless defined otherwise.

Liquefaction experiments were performed on a 150 ml scale in 500 ml stainless steel reactors. Corn ground using a hammer mill and passed through a sieve (attached to hammer mill) has particle size less than or equal to size of hole of sieve. The sieve size through which samples were ground is referred to as the grind size of corn. These samples were made to separately undergo liquefaction to study impact of particle size on AML formation. Slurry was prepared at a given percentage (w/w) solids content on dry basis with ground corn and deionized water. Slurry pH was adjusted based on recommendations of enzyme supplier for thermo stable α-amylase being used, using 10*N* sulfuric acid. The α-amylase was added to slurry based on enzyme dosage recommended by the manufacturer (0.024 to 0.03% of solids). Liquefaction was performed at temperatures of 85 or 105ºC, over 90 min with a heating and cooling rate of 3ºC/min, in a Labomat incubator (Labomat BFA-12, Werner Mathis AG, Switzerland) with continuous agitation.

To study the effect of particle size, corn ground in hammer mill passed through three sieves of size 0.5, 1.5 and 2.5 mm was collected. Corn grind size used in the industry is 2.3 mm. However, with the advent of GSHE enzymes, ethanol plants are using finer grind sizes. Thus, to cover all particle size of corn, said sieve sizes were chosen. Experiments to determine the effects of solids content were carried out by preparing slurry at 25, 32 and 34% (w/w). Industrial dry grind process uses 32 to 34 % slurry solids content. However, earlier a more dilute slurry was used. Chosen slurry content covered all of these. Action of α-amylase was studied by using AA1, AA2

and AA3 separately, and pH was adjusted to 5.1, 5.2 and 5.0, respectively, as recommended by the enzyme manufacturer. Effects of liquefaction temperature on AML formation was studied by performing liquefactions comparing different solids content (w/w) % and α-amylases at 85 and 105ºC.

Liquefaction temperature was increased to 135ºC to determine impact of breaking AML on available starch percentage (w/w) in liquefact, because AML are thermally unstable and break at temperatures of 100 to 135ºC (Kugimiya et al., 1980; Nebesny et al., 2002). Liquefaction used AA1 and 32% (w/w) solids content on dry basis but with an added step of ramping up temperature to 135ºC for 5 min after completion of regular liquefaction at 85ºC.

Low temperature liquefaction experiment used AA4, which is the α-amylase part of GSHE, at 60ºC and 32% (w/w) solids content on dry basis. The slurry pH was adjusted to 5.0 with addition of recommended dosage (0.03% of solids) of AA4. This was followed by hydrolysis of liquefact for 72 h at 32ºC using GA2, which is the glucoamylase part of granular starch hydrolyzing enzyme as per the optimal dosage after lowering the pH to 4.3. Sodium azide (0.02%) solution was added to prevent fermentation.

Pure starch liquefaction was performed using soluble starch from potato (Fisher Scientific, Waltham, MA). To confirm formation of AMLs a 5% linoleic acid (Sigma-Aldrich, St. Louis, MO) solution in 99% ethyl alcohol was added to replicate lipid content present in corn, as the oil content analysis of corn revealed linoleic acid as the dominant fatty acid (50% of total fatty acid content in corn) (Beadle et al., 1965).

Table 3.1. Liquefaction experiments that were conducted to observe impact of different liquefaction parameters on AML formation.

	Sieve size (mm)	Enzyme	pH	Temperature (ºC)	Solids content %
Effect of particle size	0.5	AA1	5.1	85	32
	1.5	AA1	5.1	85	32
	2.5	AA1	5.1	85	32
Effect of solids content % (w/w)	0.5	AA1	5.1	85	25
	0.5	AA1	5.1	85	32
	0.5	AA1	5.1	85	34
	0.5	AA1	5.1	105	25
	0.5	AA1	5.1	105	32
	0.5	AA1	5.1	105	34
Effect of α-amylase	0.5	AA1	5.1	85	32
	0.5	AA2	5.2	85	32
	0.5	AA3	5.0	85	32
	0.5	AA1	5.1	105	32
	0.5	AA2	5.2	105	32
	0.5	AA3	5.0	105	32

All experiments were carried out in a Labomat incubator for 90 min plus time taken to reach temperature.
Liquefact samples collected at the end of the experiments were dried in aluminum cans at 49ºC.
Each liquefaction experiment run was carried out in duplicate.

3.2.3 Conventional dry grind and GSHE process

Conventional dry grind process was performed using ground corn on a 150 ml basis with 32% (w/w) dry basis slurry solids content (Kumar et al., 2018). After liquefaction, liquefact pH was adjusted to 4.1 as per recommendation from manufacturer and simultaneous saccharification and fermentation (SSF) was performed by adding recommended dosage of glucoamylase GA1, urea (0.2 ml of 50% w/v solution) and yeast inoculum (1 ml of 5 g yeast dispersed in 25 ml of deionized water). Liquefact was fermented at 32ºC for 72 h in an incubator (New Brunswick Innova 42R Incubated Shaker, Eppendorf, CT) with continuous agitation of 150 rpm. Fermentation samples were taken at regular intervals to monitor fermentation and were analyzed for ethanol, glucose and other SSF intermediates.

GSH corn ethanol process was carried out on a 150 ml basis in stainless steel reactors. Slurry was prepared at 32% (w/w) dry basis solids content and pH was adjusted to 4.2 as recommended for the GSH enzyme by the manufacturer. Addition of GSHE (135 µl) was done along with 0.3 ml urea and 1.5 ml yeast inoculum. GSHE fermentation was carried out for 72 h at 32ºC in an incubator (New Brunswick Innova 42R Incubated Shaker, Eppendorf, CT) at 150 rpm.

After fermentation, reactors were placed in an 85ºC water bath kept in a chemical fume hood for 2 h to evaporate ethanol. Whole stillage was poured into aluminum pans to dry in a convective oven at 49ºC for 48 h. DDGS samples were ground and stored at 4ºC and the moisture content was measured.

3.2.4 Starch and lipid measurement

Lipid content and fatty acid profile analyses of corn, liquefact and DDGS samples were determined by the Illinois Crop Improvement Association (Champaign, IL). Measurement was made by near infrared transmission (Foss GrainSpec, Foss Food Technology, Eden Prairie, MN).

Starch content was measured using thermally stable, high performance α-amylase and amyloglucosidase (Baur and Alexander, 1979; McCleary et al., 1994). A rapid total starch enzymatic test procedure (Megazyme, Bray, Co. Wicklow, Ireland) was used (AACCI Method 76-13.01, AACCI, 2020). The assay used was specific for alpha glucans (starch, glycogen, phytoglycogen and nonresistant maltodextrins). Starch hydrolysis was carried out in 15 ml culture tubes. NaOH treatment was carried out by shaking in a rack kept in an incubator (New Brunswick Innova 42R Incubated Shaker, Eppendorf, CT) at 300 rpm for 15 min; sample tubes were vortexed at 5 min intervals. After enzyme action had broken down starch into glucose (30 min incubation), samples were mixed by inversion. Samples were centrifuged at 13,300 rpm for 10 min (Thermo Sorvall Legend Micro 17R, Fisher Scientific, Waltham, MA) and supernatant was filtered through 0.2 µm syringe filters into HPLC vials. Glucose content in sample was measured and adjusted in comparison to the glucose content in control sample (pure maize starch) to calculate starch % (w/w).

Starch complexed and bound to lipid in crystalline structures is resistant to enzyme hydrolysis and hence would not be detected by the standard enzymatic starch determination methods. AMLs have high dissociation temperatures of 100 to 135ºC; therefore, heating in boiling water cannot disperse these resistant starches (Kugimiya et al., 1980; Nebesny et al., 2002). DMSO is a hydrogen bond acceptor and a powerful solvent that can dissociate starch single and double helices and hence is used to dissolve AML to free starch molecules that are susceptible to

enzymatic hydrolysis (Srichuwong and Jane, 2011). Starch was solubilized in ethanol and dispersed in 2 ml of 90% DMSO solution. Sample tubes were kept in a beaker containing boiling water. Samples were agitated by a magnetic stir bar along with intermittent vortexing (5 min intervals) to mix samples for 30 min. Lipid dissolution and release of starch was followed by pH adjustment using sodium acetate buffer and enzymatic hydrolysis, using standard procedure (AACC International Method 76-13.01, AACCI, 2020).

3.2.5 Characterization of amylose lipid complexes

To identify liquefaction parameters responsible for higher AML formation during high temperature liquefaction (Nebesny et al., 2005), liquefact samples were compared for available starch and estimated total starch and the difference between the two values was recorded as the AML content. Available starch in sample is the starch available for enzymatic cleavage and breakdown to fermentable sugars and thus available for fermentation uptake by yeast. This was the starch content indicated by standard enzymatic starch quantification method. Estimated total starch was the starch value measured using the enzymatic hydrolysis of sample dispersed in DMSO at high temperature (>100ºC).

3.2.6 Differential scanning calorimetry

A DSC study was carried out to observe the thermal transitions in corn due to liquefaction and to confirm amylose lipid complex formation. For each DSC run 1 to 2 mg of sample was mixed with 12 µl water in a hermetic pan and was heated from 25 to 120ºC at a rate of 10ºC/min in a DSC (DSC Q2000, TA instruments, DE) (Kugimiya et al., 1980). To observe the exothermic thermal transition in corn liquefaction, ground corn sample was added with 12 µl solution of AA1 α-amylase diluted in water to maintain required dosage. The thermogram obtained was

compared to one obtained from that of pure maize starch. In accordance with prior studies (Kugiyama et al., 1980) two more samples, one with pure starch and lysolecithin from egg yolk (Sigma-Aldrich, St. Louis, MO) and another sample with pure maize starch and linoleic acid were observed for thermal transitions. Lysolecithin was chosen to replicate the experiment in prior study and linoleic acid was chosen as it was the most available AML forming fatty acid detected in corn samples.

3.2.7 Data analysis

Both dry grind and GSHE processes were carried out using three replicates. Starch content measurements of corn and DDGS used three determinants. All liquefaction experiments to determine the effect of liquefaction parameters were replicated twice. Starch content was measured with two determinants to obtain four total data points for each experimental treatment. Percentage starch available and total starch in samples were compared using one-way ANOVA and Tukey's test in R (R Core Team, 2020), to detect significant differences ($P < 0.05$). Two-way ANOVA statistical analyses were used to detect interactions among changing parameters simultaneously on AML content.

3.3 Results and discussion

3.3.1 Reduction in available starch

Conventional dry grind process and GSH enzyme process studies were carried out for comparison of AML formation. Since amylose bound in AML is inaccessible to the action of amylases and does not break into oligomers during liquefaction, it should end up in the DDGS. Hence, DDGS recovered from each process was analyzed for residual starch using standard procedure. Despite having similar final ethanol concentration (16.8 and 17.1% v/v for

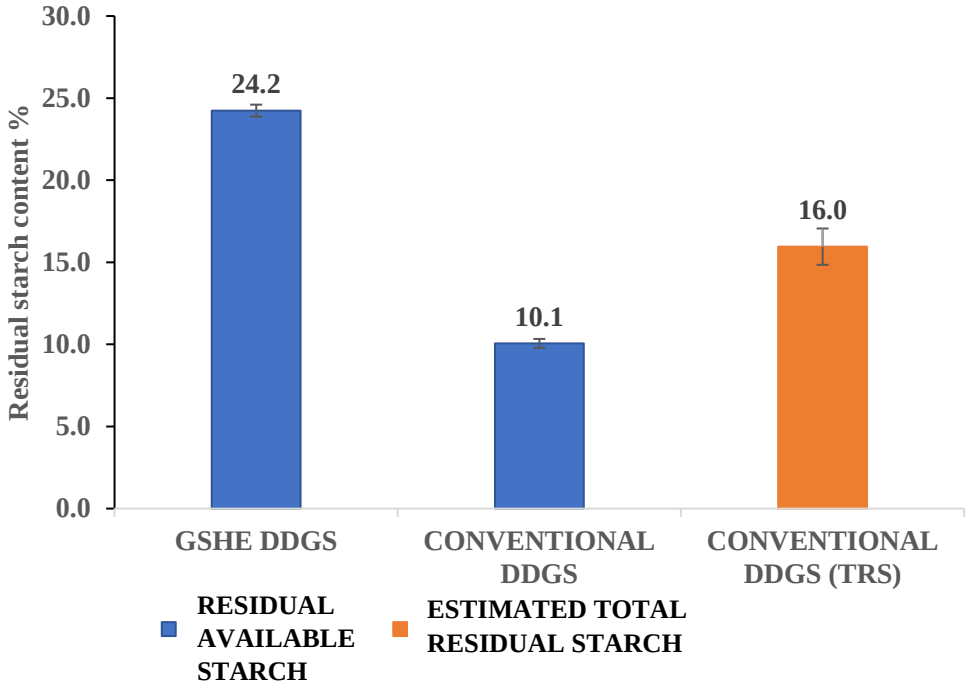
conventional dry grind and GSHE processes, respectively), residual starch content measured in DDGS recovered from GSHE process (24.2%) was higher than that from conventional process (10.1%) (Figure 3.1). Since the starch in AML is inaccessible to the enzymes, it cannot be determined using the enzymatic starch quantification assays, since these assays determine only available starch. A more accurate estimation of total residual starch would be to consider the resistant starch bound to lipid complexes, estimating them by breaking the AML to release starch bound to fatty acids. AMLs are thermally unstable at ≥ 100ºC (Kugimiya et al., 1980); by raising sample temperature, the fatty acids in the lipids can be released, making the starch available for determination using enzymatic assays.

DDGS recovered from conventional dry grind process was analyzed for total starch by dissolving the sample in DMSO and hydrolyzing using enzymes to determine accurately additional starch bound to lipids (Srichuwong and Jane, 2011). Total starch estimated using this method (16.0%) was higher than the available residual starch estimated using the standard enzymatic assay.

Available starch content in ground corn slurry was determined as 73.4% (Table 3.2). In comparison, all liquefact samples from high temperature liquefaction (≥85ºC) had lower available starch %. Liquefact obtained after liquefaction of corn slurry at 85ºC, using AA1 was found to have 61.8% starch (w/w). The decrease in measurable available starch is attributed to the AML formation. Liquefact samples show available starch content of 64.5% and 64.9% (w/w) when liquefaction temperature was raised above 100ºC, (Table 3.2) which are similar, but higher than starch available in liquefact sample from liquefaction carried out at 85ºC.

GSHE is a mixture of α-amylase and glucoamylase which can hydrolyze starch directly into fermentable sugars without the need for starch gelatinization (Sharma et al., 2007). The

liquefaction step in the conventional dry grind process is conducted at a high temperature (85ºC) to gelatinize the starch, and the α-amylase used have an optimal usage temperature of 80 to 110ºC. However, the GSH process can be carried out optimally at a low temperature of 32ºC.



TRS – Total residual starch calculated by DMSO dispersion of sample at ≥ 100ºC.

Figure 3.1. Residual starch content in DDGS measured by standard enzymatic procedure is different for GSHE and conventional dry grind process.

AMLs are formed due to high temperature liquefaction (Lee et al., 2020; Nebesny et al., 2005), and since GSH process is carried out at 32ºC; therefore, the GSH process is expected to contain higher measurable starch at the end of the process. The GSHE hydrolysis of corn, using AA4 and GA2 to completely hydrolyze starch, yielded a liquefact sample with an available starch of 69.8% (w/w). This is much higher than available starch in high temperature liquefaction samples (Table 3.2). There is reduction of starch content due to liquefaction which likely is due to AML

formation. AML formation is the reason for the decrease in the starch that can be broken down into sugars and made available for yeast uptake for increased ethanol production.

3.3.2 Impact of liquefaction parameters on available starch

To understand the breakdown of AML for improved ethanol yields in the downstream process, it is important to understand their formation with respect to liquefaction parameters such as corn particle size, solids content, liquefaction temperature and action of α-amylase. Estimated total starch recorded by enzymatic hydrolysis of liquefact sample dispersed in DMSO (Srichuwong and Jane, 2011) was similar across varying parameters in study of single varying liquefaction parameter. Hence, difference in total estimated starch and available starch were due to varying AML content in each sample (Figure 3.2).

Table 3.2. Comparison of available starch content.

	Sieve size (mm)	Enzyme	Temperature (ºC)	Solids content %	Available starch %
Liquefact	0.5	AA1	85	32	61.8 ± 0.58^{d}
	0.5	AA1	105	32	64.5 ± 1.86^{c}
	0.5	AA1	135	32	64.9 ± 1.43^{c}
	0.5	AA4/GA2[1]	60/32	32	69.8 ± 0.82^{b}
Corn					73.4 ± 1.67^{a}

[a,b,c,d] Least significant difference $P < 0.05$
[1]AA4/GA2 are the GSHE enzymes

Increasing corn grind size from 0.5 to 2.5 mm resulted in a decrease in the AML formation during liquefaction (Figure 3.2a), as is observed by the decrease in the difference between available starch and total starch estimated (3.46% for ground corn passed through 0.5 mm sieve compared to 1.00% for 2.5 mm sieve). This could be attributed to the fact that corn with larger

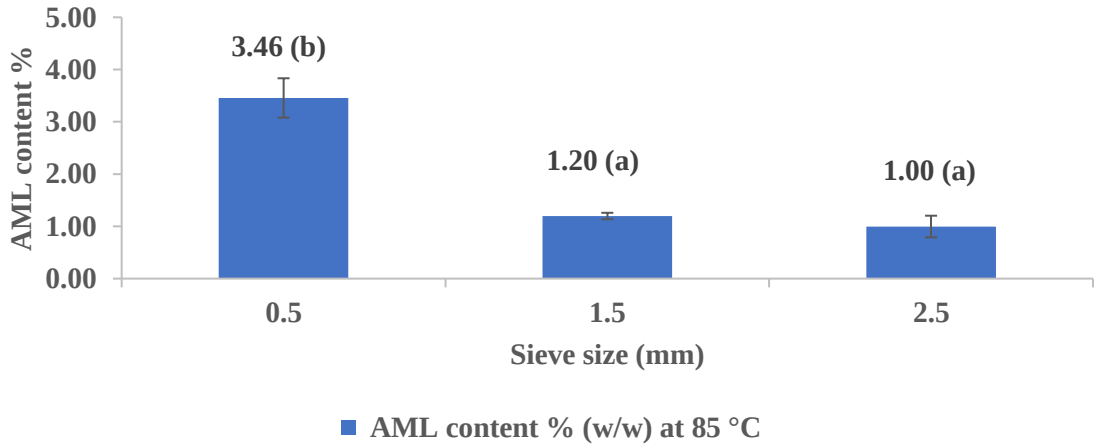
grind size will have larger particle size and have large enough starch granules. This would lower interaction of starch with fatty acids preventing complex formation. Finer grind size of corn was shown to increase the starch availability for enzymatic hydrolysis but also increases susceptibility to AML formation. Finer grind size could also imply increase in available free fatty acid from ground germ. Increase in available fatty acids would lead to an increase in AML content.

No consistent trend was observed for increasing solids content during liquefaction (Figure 3.2b). The AML content was observed to increase when solids content was increased from 25 to 32%, from 2.13 to 3.46%. However, with a further increase of solids content to 34%, AML content decreased to 1.62%. Increasing the liquefaction temperature from 85 to 105ºC resulted in a decrease in the AML content (and increase in the available starch percentage in liquefact), irrespective of the percentage solids content. The mean of difference between AML content for all three solids contents is 0.61% when liquefaction temperature is increased to 105 ºC from 85 ºC.

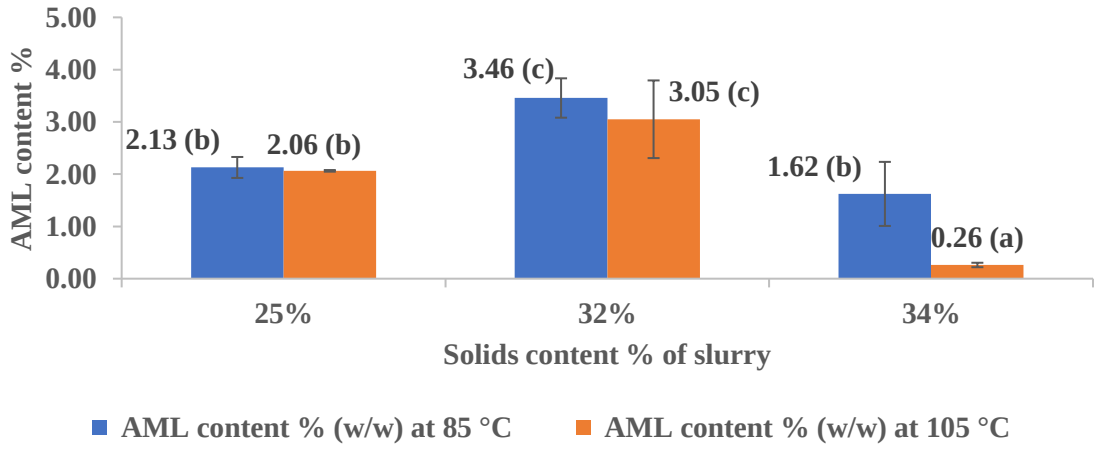
Also, using two-way ANOVA statistical analysis, it was found that that solids content and temperature of liquefaction (85 and 105ºC) were interacting factors affecting AML content. Thus, the difference in AML content in liquefact due to change in solids content of liquefaction, also depended on change in liquefaction temperature.

Action of α-amylase during liquefaction is responsible for breaking down starch to long chain oligomers and affects available starch content. All three enzymes had similar enzyme activity (11000 to 13000 µmol maltose/ml enzyme) and resulted in similar differences among available starch and estimated total starch when dispersed in DMSO for liquefaction at 85ºC (Figure 3.2c).

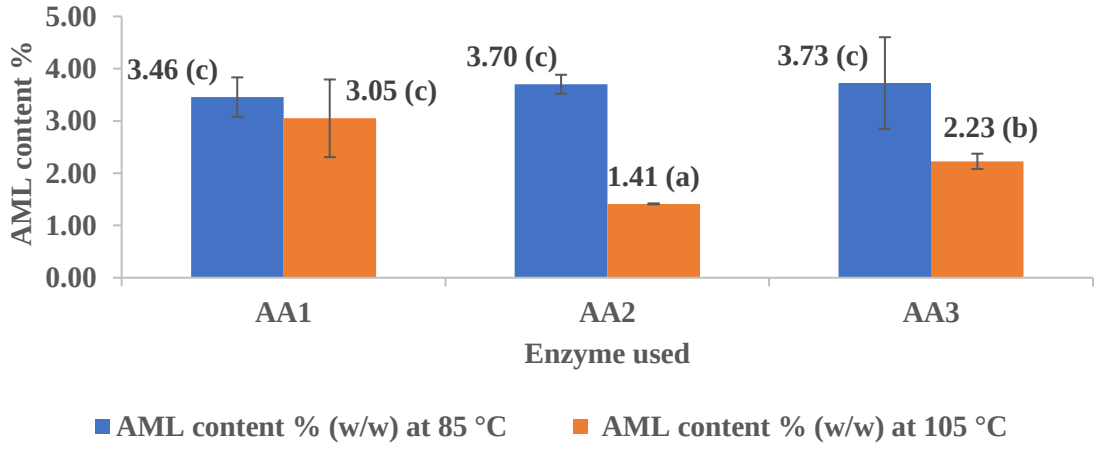
2a



2b



2c



[a,b,c] Least significant difference $P < 0.05$

AML content is difference between estimated total starch by dispersion of sample in DMSO and available starch by standard enzymatic assay.

Figure 3.2. Impact of liquefaction parameters on AML content in liquefact samples.

However, AA2 had lower AML content with a difference of 1.41% compared to 3.05 and 2.23% for AA1 and AA3, respectively, when liquefaction temperature was increased to 105ºC. Thus, action of different α-amylase affects AML content differently. Hence, it is important to gauge impact of enzyme used on AML content and available starch in liquefact before use.

Increasing the liquefaction temperature to 105ºC resulted in lower AML content across liquefact obtained from all three enzymes studied. Interaction between enzyme used for liquefaction and liquefaction temperature, did not result in different results. Irrespective of whichever of the three enzymes was used, AML content decreased with increase in liquefaction temperature. Moreover, AML content in liquefact recorded due to action of specific enzyme during liquefaction, was the same irrespective of liquefaction temperature. These two factors were additive.

3.3.3 Role of liquefaction and corn lipids in AML formation

To study further the role of liquefaction and interaction between starch and fatty acids at high temperatures (85ºC), commercial soluble starch from potato was processed at high temperature and agitation, with similar conditions as liquefaction. A 32% solids content slurry taken through high temperature of 85ºC with and without AA1, resulted in no change in percentage available starch calculated compared to original starch slurry (Table 3.3). Starch, by itself, remains unaffected by high temperature during liquefaction and does not lower starch content available for conversion to sugars; the complexes are formed due to interaction of starch with other components of corn.

AML in corn liquefaction would be formed due to interaction between gelatinized starch and fatty acids present in corn. Ground corn used in this study had 4.2% oil content on dry basis. Fatty acid profile showed 54.0% linoleic acid (Table 3.4). Other fatty acids, like oleic acid,

stearic acid and palmitic acid which have an affinity to form AMLs also are present in corn (Cervantes-Ramírez et al., 2020; Kaur and Singh, 2000; Nebesny et al., 2002).

Table 3.3. Role of liquefaction temperature and lipids in AML formation.

	Enzyme	Temperature (ºC)	Solids content %	Available starch %
Starch				81.4 ± 0.18[a]
Treated	N/A	85	32	80.2 ± 0.42[a]
starch[1]	AA1	85	32	80.8 ± 0.76[a]
Starch and	N/A	85	25	77.0 ± 1.11[b]
lipid[2]	N/A	32	25	81.7 ± 1.91[a]

[a,b] Least significant difference $P < 0.05$
[1]Pure potato starch slurried with deionized water and agitated at 85ºC for 90 min
[2]Mixture of pure potato starch and linoleic acid slurried with deionized water

High temperature agitation of pure potato starch with lipids (linoleic acid) at 85ºC led to reduction in available starch content from 81.4 to 77.0% (Table 3.3). The starch lipid mixture was left to mix and agitated at 32ºC for 3 h but showed no change in calculated available starch content. Thus, presence of lipids as well as high temperature (85ºC) are responsible for reduction in available starch, and this is attributed to interaction of fatty acids with starch which gelatinizes at high temperature of more than 80ºC to form AMLs.

3.3.4 Confirmation of amylose lipid complex

Starch behavior under increasing temperature is studied using differential scanning calorimetry (DSC) by observing the enthalpy changes over a range of temperature. Thermal phase transitions

Table 3.4. Lipid profile of corn sample used for study.

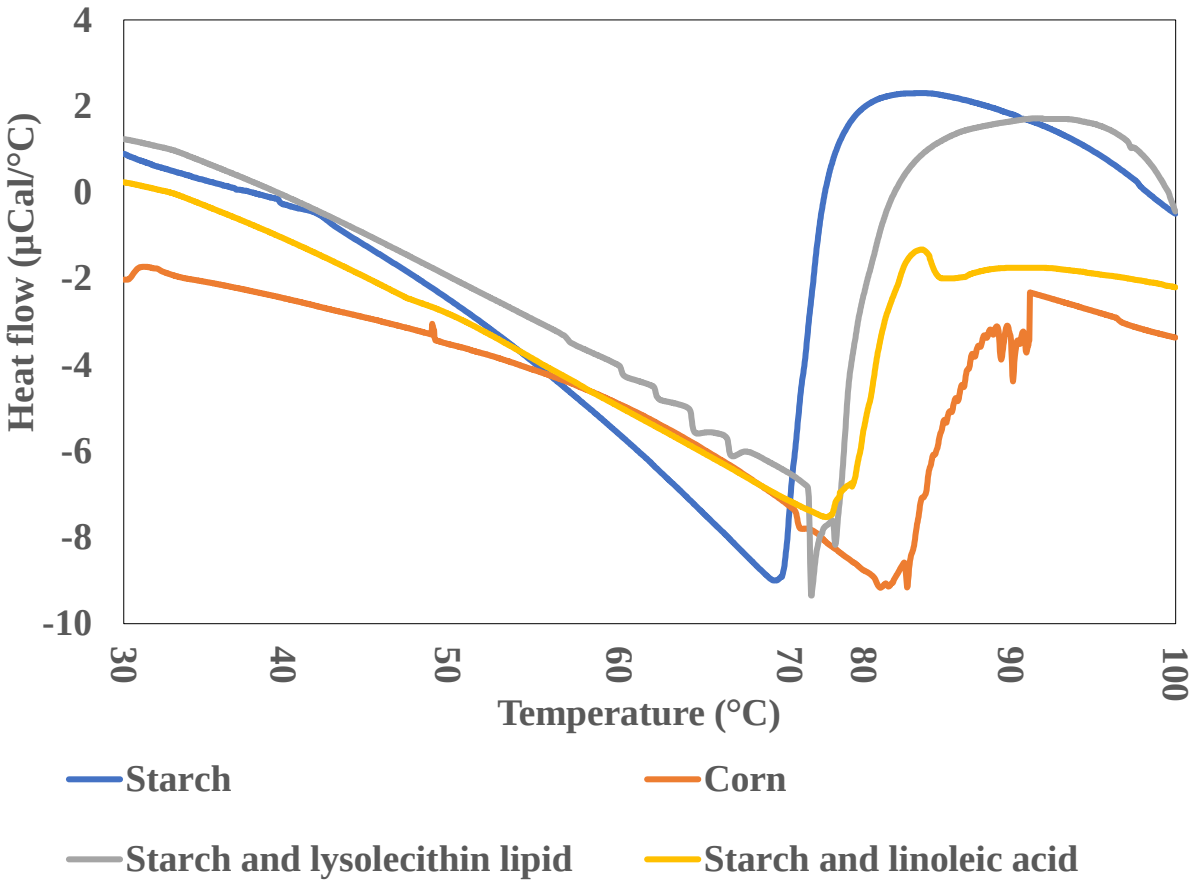
Fatty acid	Oil content (%)
Palmitic	13.80
Stearic	1.73
Oleic	25.96
Linoleic	54.04
Linolenic	1.40

% w/w dry basis

that starch undergoes on heating in the presence of water resulted in an endothermic reaction which was indicative of gelatinization. Another exothermic transition also was observed at temperatures higher than those at which gelatinization occurs, in the presence of lipids. This exothermic transition was indicative of the formation of AMLs (Kugimiya et al., 1980; Nebesny et al., 2002).

DSC thermograms of corn liquefaction were compared with two other samples, one containing pure corn starch and another with corn starch and linoleic acid (Figure 3.3). The samples were processed with water and AA1 enzyme added to the recommended dose. The exothermic notch indicating AML formation which releases energy were found only in samples containing lipid as well as whole ground corn. This exotherm was more visible in stark contrast with the smooth gelatinization endotherm of pure starch. The figure was truncated at 100ºC where another endotherm is observed indicating AML breaking due to thermal instability. AML formation only occurred once gelatinization of starch had occurred and at temperatures higher than 70ºC. Furthermore, comparison of exotherms is an indicator of how varying fatty acids have different

energy of formation as is seen in the thermogram of the ground corn sample (Nebesny et al. 2002).



Positive change is exothermic and negative change is endothermic.
Pure maize starch (95%) with 11.3% moisture was used.
Sufficient water was added along with sample in hermitic pans to allow proper heat transfer.

Figure 3.3. Comparative thermograms of corn, pure starch and starch lipid standards.

3.4 Conclusions

High temperature liquefaction step in the conventional dry grind ethanol process leads to formation of amylose lipid complexes (AML). They are formed due to interactions among gelatinized starch and corn lipids in an exothermic reaction. These complexes lead to formation

of resistant starch that are not available to α-amylase action and thus reduce available starch in liquefact. Formation of AMLs and consequent reduction in available starch can be controlled by considering the effects of liquefaction parameters. Larger particle size, raising liquefaction temperature to more than 100ºC and use of enzyme whose action is unfavorable for AML formation can help reduce AML formation. This in turn will increase starch available for breakdown during enzymatic hydrolysis and saccharification.

Chapter 4

COMPARISON OF TWO LIQUEFACTION TREATMENTS TO LOWER AMYLOSE COMPLEX FORMATION

4.1 Introduction

Available starch in postliquefaction solids is impacted by formation of amylose lipid complexes (AMLs) during liquefaction. However, combined effects of parameters that lower AML formation on the dry grind process is unknown. AMLs are resistant to enzymatic breakdown (Haralampu, 2000; Seneviratne, 1991); and AML content varies with liquefaction parameters (Chapter 3). AML content can be measured by observing the difference between total starch estimated by DMSO treated sample and available starch measured from standard enzymatic starch estimation method. AML content in liquefact may help explain the lack of relationship between starch content in corn and starch utilized for ethanol production.

Residual starch in distillers' dried grains with solubles (DDGS) is dependent on available starch in liquefact remaining after fermentation (Chapter 3). Temperature, enzyme used, solids content and corn particle size affect liquefied slurry by changing AML content. Differences in AML content were indicative of variation in available starch for fermentation.

AML content in liquefact was measured using two liquefaction treatments followed by simultaneous saccharification and fermentation. The two liquefaction treatments were; 1. ground corn passed through 0.5 mm sieve, at 32% (w/w) solids content on dry basis, using AA1, at 85ºC (T1) and 2. ground corn passed through 1.5 mm sieve, at 34% (w/w) solids content on dry basis, using AA2, at 105ºC (T2). The specific objective of this study was to compare AML content in DDGS for the two treatments. These two treatments were chosen in order to show the difference

in AML content that can be brought about by choosing the correct parameters that lower AML formation during liquefaction.

4.2 Materials and methods

4.2.1 Materials

Ground corn was obtained from a commercial dry grind ethanol plant and stored at 4ºC in a plastic bucket. The corn was further ground at 500 rpm in a laboratory hammer mill (Retsch SK100, Glenn Mills, Clifton, NJ) and screened through a sieve. Corn sample was stored in Ziploc bags at 4ºC until analysis. Moisture content of ground corn sample and DDGS was measured by drying weighed samples in a convection oven at 105ºC overnight (AACCI Approved Method 44-15.02, AACCI, 2020).

The α-amylases and glucoamylases were commercial enzymes used in dry grind process. The study was carried out using two thermostable α-amylases, AA1 and AA2, having activities of 11648 and 13501 µmol maltose/ml enzyme, respectively. The amyloglucosidases used for saccharification, GA1, had an activity of 18197 µmol glucose/ml enzyme.

Liquefaction experiments were carried out at optimal pH (5.0 to 5.2) provided by enzyme manufacturers. Ethanol Red conventional dry yeast was used for fermentation (Fermentis-Lessaffre Yeast Corporation, Milwaukee, WI).

4.2.2 Dry grind process

Corn obtained from a commercial dry grind ethanol plant was ground at 500 rpm in a laboratory hammer mill (Retsch SK100, Glenn Mills, Clifton, NJ), and passed through a 0.5 mm and 1.5 mm sieve to obtain two samples (T1 and T2, respectively). Liquefaction was performed on a 250

ml scale in 500 ml stainless steel reactors in sets of four to add up to a total volume of 1 L. For treatment 1, slurry was prepared at 32% (w/w) solids content on dry basis with ground corn and deionized water (T1). For treatment 2, slurry solids content was 34% (w/w) on dry basis (T2). The pH of the slurry was adjusted based on recommendation of enzyme supplier for thermo stable α-amylase being used, using 10*N* sulfuric acid. The α-amylase was added to slurry based on enzyme dosage recommended by the manufacturer (0.024 to 0.025%). Liquefaction was performed at 85ºC (T1) and 105ºC (T2), over 90 min with a heating and cooling rate of 3ºC/min, in a Labomat incubator (Labomat BFA-12, Werner Mathis AG, Switzerland) with continuous agitation.

After liquefaction, liquified slurry was prepared for simultaneous saccharification and fermentation (SSF). SSF was carried out in a benchtop bioreactor (New Brunswick Bioflo/Celligen 115, Eppendorf, Enfield, CT) with a 2 L volume capacity tank. The total liquefact of 1 L was poured into the tank. The pH probe was calibrated using standard solutions of pH 4 and 7 prior to SSF. The motor pumps that cycled acid and base to maintain pH were adjusted to operate at 80% (base pump) and 20% (acid pump), respectively. The bioreactor was set up for a batch process and control was used to maintain pH at 4.3 using 10*N* solutions of sulfuric acid and sodium hydroxide. Once set pH was reached, recommended dosages of GA1, urea (0.2 ml of 50% w/v solution) and yeast inoculum (1 ml of 5 g yeast dispersed in 25 ml of deionized water) were added to the reaction tank through a port on the headplate of the reactor. The reaction mixture was agitated continuously using submerged impellers. To maintain homogenous conditions in reaction volume, agitation for slurry with 32% solids content was carried out at 400 RPM, and for slurry with 34% solids content was carried out at 1200 RPM.

Reaction temperature was maintained at 32ºC using a heating jacket. SSF of liquefact was carried out for 72 h.

Any unused ports were plugged to prevent evaporative loss of ethanol. An exhaust condenser was installed and connected to a chiller unit (PolyScience, Niles, IL), which maintained flow of cold water at 2ºC, preventing loss of ethanol while regulating reactor temperature. After completion of fermentation, entire reaction volume was poured into weigh boats and left to dry at in a convective oven at 49ºC. The obtained DDGS were ground and stored at 4ºC; moisture content was measured.

4.2.3 Measurement of starch in DDGS

Residual starch content in DDGS was measured using a thermo stable, high performance α-amylase and amyloglucosidase as described in Chapter 3 (Baur and Alexander, 1979; McCleary et al., 1994). The rapid total starch enzymatic test procedure (Megazyme, Bray, Co. Wicklow, Ireland) was followed (AACCI Method 76-13.01, AACCI 2020).

Total starch in DDGS was estimated using Dimethyl sulfoxide (DMSO) to dissolve amylose lipid complex crystals to free starch molecules that are susceptible to enzymatic hydrolysis (Srichuwong and Jane, 2011). The procedure followed was the same as the one described in Chapter 3.

4.2.4 Data analysis

Dry grind treatments were performed twice for each treatment. Starch content measurement of DDGS was done in triplicate. Percentage available starch and total residual starch in samples were compared using one way ANOVA and Tukey's test in R (R Core Team, 2020) to determine significant ($P < 0.05$) differences.

4.3 Results and discussion

Residual starch content in DDGS is indicative of starch that was not taken up by yeast during the dry grind process and is left over in the solids. This includes enzyme resistant starches. AML bound starch content is not broken down during liquefaction and ends up in DDGS. The DDGS recovered from each treatment (T1 and T2) were analyzed for residual available starch and total starch including AML bound resistant starch.

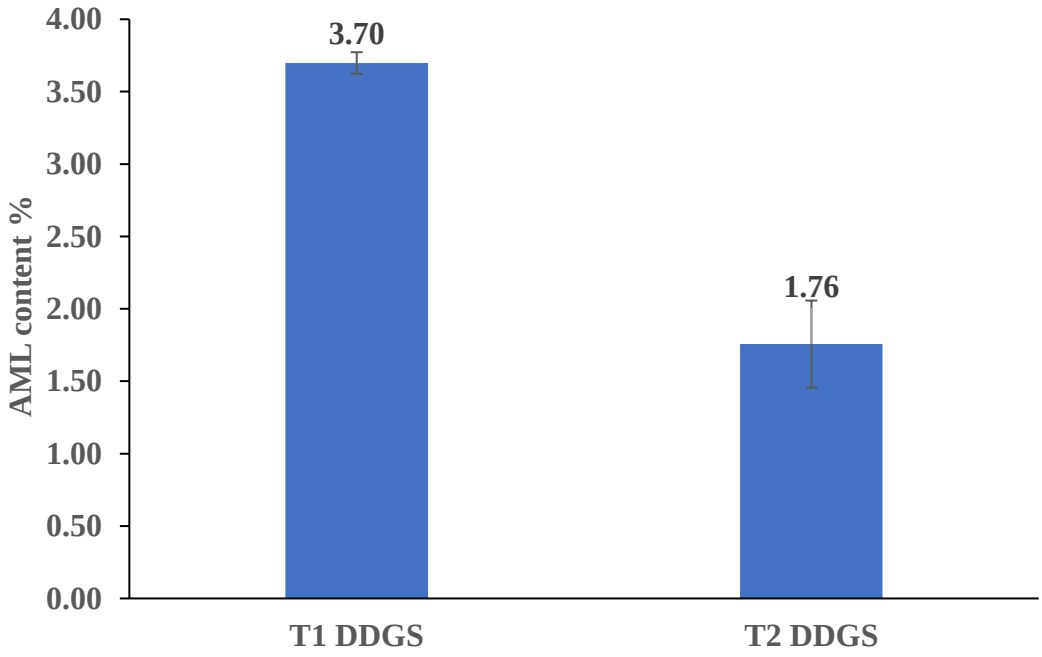


Figure 4.1. Comparison of the AML content in the DDGS extracted from two liquefaction treatments.

The AML content, found by the difference in residual available starch and residual total starch in DDGS, was higher for treatment 1 than treatment 2 by 1.94% (Figure 4.1). A combination, of increasing liquefaction temperature, increasing particle size of corn and a higher solids content in slurry in treatment 2 resulted in lower AML formation.

4.4 Conclusions

Resistant starch in AML formed during high temperature liquefaction in the conventional dry grind process ends up in DDGS. Resistant starch in the form of AML is unaffected by SSF. The conditions identified in Chapter 3 that lower AML content were, a liquefaction temperature higher than 100ºC, higher solids content of 34% and higher corn particle size. The combined impact of these conditions during liquefaction decreases AML content.

Chapter 5

RECOMMENDATIONS FOR FUTURE WORK

Resistant starch in AMLs ends up in DDGS and the AML content in liquefact is found to be similar to that in DDGS. Liquefaction conditions that lower AML formation and reduce available starch for enzymatic hydrolysis by almost 2% have been identified. Having confirmed presence of AML, the following are suggestions for further analyses:

1. Increase in ethanol yield can be determined by carrying out liquefaction as per conditions known to lower AML formation, followed by SSF under similar conditions as control.
2. The impact of this study on the industry, accounting for higher solids content and higher temperature can be identified by conducting a technoeconomic study.
3. Starch availability was shown to be impacted by AML formation. Action of adding lipases and other lipolytic enzymes alongside amylolytic enzymes which could break these AML during SSF and free up starch for enzymatic hydrolysis could be studied.
4. Impact of increasing liquefaction time at temperatures higher than 100ºC which would lead to an increased degradation of AMLs can be studied.
5. AMLs affect viscosity of starch liquefact. A comparative rapid viscosity analysis (RVA) study of liquefacts obtained from conditions with lower and higher AML formation can be carried out to determine its impact on the industry.

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